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Myeloperoxidase associates with degenerative remodeling and rupture of the saccular intracranial aneurysm wall

Running head: Myeloperoxidase in intracranial aneurysms

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Abstract

Rupture of a saccular intracranial aneurysm (sIA) is often fatal. Thus, early detection of rupture-prone sIAs is vital. Myeloperoxidase (MPO), derived mainly from neutrophils, associates with sIA rupture, and therefore its role in sIA pathogenesis warrants further studies. We analyzed MPO and its association with other histological markers in 36 (16 unruptured and 20 ruptured) sIA samples by immunohistochemistry. MPO was present in all studied sIAs, and its expression associated with wall inflammatory cell infiltrations ($r=0.50$, 0.63, and 0.75, all $p \leq 0.002$), degenerative remodeling ($p=0.002$) and rupture ($p=0.003$). MPO associated strongly with the presence of organized luminal thrombi ($p<0.001$), which also stained positive for MPO. Polymorphonuclear MPO^+ cells were detected in the sIA walls, indicating neutrophils as MPO-source. MPO correlated strongly with accumulation of oxidized lipids ($r=0.67$, $p<0.001$) and loss of smooth muscle cells ($r=-0.68$, $p<0.001$), suggesting that MPO is a relevant source of oxidative stress leading to cell death in the sIA wall. Furthermore, MPO associated with erythrocyte fragmentation ($r=0.74$, $p<0.001$) and iron deposition ($p=0.041$), two outcomes known to amplify MPO-dependent oxidative stress. Taken together, these results suggest that MPO associates with degenerative remodeling predisposing to sIA wall rupture and may serve as a biomarker of a rupture-prone sIA wall.

Keywords

Aneurysm, atherosclerosis, inflammation, myeloperoxidase, oxidative stress, thrombus

Introduction

Rupture of a saccular intracranial aneurysm (sIA) causes intracranial hemorrhage with high mortality (40-50%) (1). Importantly, unruptured sIAs are common in the population (2-3%) (2, 3), and a fraction of these sIAs rupture (4). Therefore, understanding sIA pathobiology is essential for development of new methods that better distinguish and treat sIAs of high rupture risk. Rupture-prone sIA walls show degenerative remodeling, chronic inflammation, lipid accumulation, and oxidative stress (5-15), contributing to the cytotoxic microenvironment in the sIA wall (15).

Myeloperoxidase (MPO) is an oxidizing enzyme mainly derived from neutrophils (16-19) and is linked with the sIA rupture (20). MPO contribution to sIA formation and rupture has also been demonstrated in a mouse model of induced sIA (21). Here we studied how the MPO expression in the human sIA wall associates with degenerative remodeling and pathological changes characteristic of ruptured sIAs.

Materials and methods

We studied a previously published histological series of 36 sIAs (16 unruptured and 20 ruptured, Supplemental Table 1) (13, 14) resected after clipping the sIA neck at the Department of Neurosurgery, Helsinki University Hospital, Finland. Clinical data were collected from patients' medical records. The Hospital Ethics Committee approved the study protocol.

The tissue samples were treated and immunohistological stainings, imaging, and analysis were performed as previously described (13, 14). Supplemental Table 2 shows the primary antibodies used in this study and their concentrations. To analyze the presence of myeloperoxidase (MPO) and matrix metalloproteinase-9 (MMP-9) in the sIA wall, localization (either intracellular or extracellular in the wall or in the thrombus) and the extent of expression were scored semiquantitatively (1, 2, 3, or 4) as described in Figure 1. We additionally defined the degree of colocalization of MPO-expressing areas with areas of apolipoprotein A-I (apoA-I; the major protein component of for high-density lipoprotein, HDL) accumulation as complete, partial, or absent.

Statistics

Data analysis was performed using IBM SPSS Statistics Software, version 21 (IBM Corporation). For categorical variables, proportions were calculated, and Fisher's exact test was used. For continuous variables, median and range were calculated, and Mann-Whitney U (MWU) test, Kruskall-Wallis (KW) multiple comparison test, and Spearman correlation tests were used. P-values <0.05 were considered significant.

Results

MPO associates with degenerative wall remodeling of sIA walls

Myeloperoxidase (MPO)-positive staining was present in all 36 studied saccular intracranial aneurysms (sIAs) and correlated with loss of smooth muscle cells (SMCs; r=-0.68, p<0.001, Spearman), a characteristic feature of rupture-prone sIAs (6, 22). Degenerated type C walls showed a more extensive presence (higher score) of MPO in the sIA wall than those from the less degenerated types A and B (Figure 2a, Table 1). The MPO score associated with sIA wall rupture (Table 1). MPO strongly correlated with matrix metalloproteinase-9 (MMP-9; r=0.81, p<0.001, Spearman), which associated with sIA wall degeneration and rupture (p<0.001, Fisher, Table 1). MPO was expressed in all fresh or organized thrombi, present in 22/36 (61%) sIAs (Figure 1c and 2b). The presence of organized luminal thrombi also associated with high MPO score (p<0.001, Fisher). In the sIA wall and thrombus, MPO was abundant both intracellularly and extracellularly. High MPO score correlated with the numbers of $CD163^+$ and $CD68^+$ phagocytes and T lymphocytes (Figure 2c-e).

MPO associates with accumulation of erythrocyte degradation products in sIA walls

The MPO score correlated with the accumulation of glycophorin A (GPA; r=0.74, p<0.001, Spearman), an erythrocyte surface glycoprotein (23), and with the presence of iron (Perl's blue –positive iron, hemosiderin; p=0.041, Fisher), known to be derived from the hemoglobin of degraded erythrocytes. High MPO score was correlated with extensive presence of heme oxygenase 1 (HO-1, r=0.52, p=0.001, Spearman), a detoxification enzyme induced by erythrocyte-derived heme and oxidative stress (24-26).

MPO correlates with lipid accumulation in sIA walls

MPO score correlated with accumulation of apolipoprotein B-100 (apoB-100); this accumulation reflects infiltration of blood plasma –derived apoB-100-containing lipoprotein particles into the sIA wall

(Figure 3a). Moreover, the MPO score correlated with oxidized, but not neutral, lipids in the sIA walls (Figure 3b-c). MPO score correlated with the expression of adipophilin, an indicator of intracellular neutral lipid accumulation ($r=0.59$, $p<0.001$, Spearman). Interestingly, the presence of high-density lipoprotein (HDL) -associated apolipoprotein A-I (apoA-I) particles also correlated with high MPO score (Figure 3d), and MPO colocalized with apoA-I partly (8/36) or completely (9/36) in 17/36 (47%) sIAs, of which 14/17 (82%) were ruptured ($p=0.013$, KW).

Association of MPO with clinical risk factors for IA rupture

A comparison of MPO score with clinical risk factors is shown in Table 2. The MPO score did not correlate with the various parameters characterizing sIA size, such as base, fundus length, or fundus width ($r=-0.17$ to -0.09, $p=0.336$-0.614, Spearman). MPO score did not associate with smoking or the PHASES (Population, Hypertension, Age, Size, Earlier SAH, and Site) (27) score for sIA rupture ($r=-0.32$, $p=0.061$, Spearman).

Discussion

In this study, we examined 36 unruptured or ruptured sIAs for the presence of MPO and found that all sIAs contained MPO. Furthermore, we demonstrated that MPO associates with histological changes of the sIA wall that are characteristic for degenerative remodeling predisposing to wall rupture.

MPO associates with degenerative remodeling and rupture of sIA walls

MPO was present not only in all studied sIAs, but its expression was also the highest in degenerated and ruptured sIA walls, thus providing strong suggestive evidence for a role of MPO in the clinically critical changes in sIA walls. Previously, Gounis et al reported the presence of MPO in three ruptured and 10/20 unruptured sIAs (20). Chu et al demonstrated increased MPO presence in sIA walls when compared with the peripheral vascular wall (21). Moreover, in the cited study (21), samples of luminal blood derived from sIA sacks showed higher MPO concentrations than those of samples derived from peripheral blood of the patients. In an angiotensin II-elastase-induced mouse model of cerebral aneurysm, the same group showed that MPO can contribute to aneurysm formation and rupture (21). Jointly, the above findings and those of the present study imply that MPO plays a role in sIA wall degeneration and rupture.

In atherosclerotic plaques, MPO causes vascular wall fragility through various mechanisms, including activation of matrix metalloproteinases (MMPs), which can degrade the extracellular matrix of the arterial wall (17). Among the several proteases expressed in sIAs (28, 29), MMP-9 is of particular interest as this protease, similar to MPO, is released by neutrophils during their degranulation (30). Importantly, released MPO activates the simultaneously released inactive pro-form of MMP-9 (31). MPO also activates the pro-forms of two other neutrophil-derived MMPs, namely those of MMP-7 and the neutrophil collagenase MMP-8 (32, 33). Accordingly, in sIAs as in atherosclerotic plaques, MPO can activate both neutrophil-derived and locally synthesized pro-MMPs, and via such activation can promote degradation of collagen and elastin fibers. In addition to an increased proteolytic catabolism of the extracellular matrix of the sIA wall, attenuation of matrix synthesis would also contribute to reduced tensile strength of the wall. Interestingly, we found that MPO expression in the sIA wall was

associated with the loss of SMCs, thereby reducing the number of these matrix-synthesizing cells in the wall. Mechanistically, the finding also suggests that MPO-derived oxidative stress contributes to the loss of SMCs (34). The presence of cytotoxic microenvironments within the sIA wall is consistent with the notion that oxidative stress is involved in sIA pathogenesis (15). Since MPO is strongly oxidizing, it may be a significant contributor of oxidative stress in sIA walls as has been shown in other vascular pathologies (16-18).

Thrombus as a source for MPO and lysed erythrocytes

We found MPO particularly widespread in the luminal thrombus. In other vascular diseases, the luminal thrombi trap polymorphonuclear leukocytes (i.e. neutrophils), which produce MPO, often further stored and delivered by macrophages (18, 34). We observed polymorphonuclear cells in the thrombi covering the sIA walls; these cells were positive for MPO immunostaining. In addition, also MPO^+ mononuclear cells were also present. Our findings implicate the thrombus, which is often a very thick layer in the sIA, as the major source for MPO in sIA. MPO expression associated strongly with increased numbers of $CD163^+$ and $CD68^+$ macrophages, which may also produce MPO (17, 35), or participate in the storage and release of neutrophil-derived and monocyte/macrophage-derived MPO in the sIA.

Erythrocytes trapped in an arterial thrombus become lysed and release hemoglobin and other biologically active components (34, 36, 37). In addition to the luminal thrombus, neovessel-derived intramural microhemorrhages are another potential source for erythrocytes (13). We showed that the accumulation of erythrocyte-specific protein GPA correlated with high MPO expression. The cytotoxic potential of MPO may contribute to lysis of the thrombus-trapped erythrocytes and also oxidize the released lipid-rich erythrocyte membranes. Furthermore, lysis of erythrocytes releases pro-oxidative hemoglobin, whose ferrous iron amplifies the pro-oxidative effect of MPO (38). Here, the presence of iron deposition was associated with high MPO expression, suggesting the presence of a strong oxidizing environment particularly in the sIAs containing both iron and MPO.

MPO may contribute to accumulation of oxidized lipids in sIA walls

We have previously shown that lipid accumulation and atherogenic changes occur in sIA walls, and that these changes associate with degenerative remodeling and wall rupture (12-14). We now observed that MPO is associated with the extent of immunostaining for apolipoprotein B-100, the protein component of all atherogenic lipoprotein particles (i.e., of those which transport cholesterol and other lipids to vascular walls) (39). Moreover, MPO associated with immunostained hydroxynonenal, which reflects the presence of oxidized lipids (40). In human atherosclerotic lesions, MPO catalyzes oxidation of lipids and lipoproteins (16-18). Therefore, MPO may also contribute to lipid oxidation also in the sIA wall.

MPO-dependent oxidation in aortic atheroma plaques is also known to cause dysfunction of the HDL-mediated cellular cholesterol clearance mechanism by oxidation of apoA-I (41, 42), the major structural and functional protein of HDL (43). MPO can specifically bind to the apoA-I component of HDL particles (44). Interestingly, MPO-bound HDL particles are detected in the blood plasma of patients with abdominal aortic aneurysms (45). Our analysis revealed that nearly half of the studied sIAs showed either complete or partial colocalization between MPO-positive and apoA-I-positive wall areas, raising the possibility that in these areas some of apoA-I might have been oxidized by MPO. Our recent findings demonstrated that intracellular lipid accumulation in SMCs (i.e., SMC foam cell formation) correlated with apoA-I-positive staining in sIA walls, and that foam cell death had occurred in degenerated and ruptured sIA walls (14). A fraction of the apoA-I did colocalize with apoE, which could have immobilized apoA-I in the extracellular matrix of the sIA wall and thus prevented it from inducing cholesterol efflux from the foam cells (14). The findings of the present study add one potential mechanism capable of inhibiting the ability of apoA-I to remove cholesterol from foam cells, namely the MPO-dependent oxidation of apoA-I. Since continuous intracellular accumulation of cholesterol may trigger death of the foam cells with ensuing inflammation in the sIA wall, dysfunctional apoA-I could potentially be a promoter of aneurysm wall rupture (14).

MPO as a potential biomarker for a fragile sIA wall

Contrary to the previous report by Gounis et al (20), in our series of sIAs MPO did not associate with established clinical risk factors for sIA rupture, such as PHASES score (27) or smoking, despite association with degenerative wall remodeling and sIA rupture. Thus, our findings suggest that the widespread presence of MPO may signify a fragile rupture-prone wall also in sIAs that lack the classical risk factors. MPO can be detected with contrast agent-enhanced MRI (46), and in blood samples drawn from sIA fundus (21). The lack of correlation between MPO and established risk factors suggests that MPO may serve as an independent biomarker to assess the risk of sIA rupture.

Conclusion

MPO is present in the sIA wall and may play a role in degenerative wall remodeling and loss of SMCs. Leukocytes in the luminal thrombus are a major source for MPO, which increases oxidative stress and accumulation of oxidized lipids in the sIA wall. As MPO associates with degenerative wall remodeling that predisposes to rupture, but not with the established risk factors for rupture, MPO may serve as an independent novel biomarker of a rupture-prone sIA.

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Figure Legends

Figure 1. Demonstrative images of myeloperoxidase (MPO) and matrix metalloproteinase-9 (MMP-9) staining patterns in saccular intracranial aneurysm (sIA) walls. To score the extent of MPO accumulation in the sIA walls, we divided the sIAs into four groups according to the pattern of positive staining (brown). **a**, Single scattered positive cells (score 1; n=5/36, 14%, arrowhead); **b**, positive staining (intracellular and extracellular) in a restricted wall area (score 2; n=11/36, 31%); **c**, positive staining (intracellular and extracellular) in a large area (but less than half of the wall area) (score 3; n=8/36, 22%, OT stands for organized thrombus); **d**, majority (more than a half) of the wall area is positively stained (score 4; n=12/36, 33%, negative control is the inset). For MMP-9, 30/35 sIAs showed positive staining, and were scored accordingly as follows: **e**, single scattered positive cells (score 1; n=12/35, 34%, arrowheads); **f**, positive staining (intracellular and extracellular) in a restricted wall area (score 2; n=7/35, 20%); **g**, positive staining (intracellular and extracellular) in a large area (but less than half of the wall area) (score 3; n=10/35, 29%). Only 1/35 (3%) of the sIAs showed MMP-9-positive staining in majority (more than a half) of the wall area (score 4). **d,** Accumulation of MMP-9-positive cells in a fresh thrombus (FT, negative control is the inset). The arrows on the right-hand margin show the wall orientation from the adventitial to the luminal side. The scale bar is 50 μm for a and e and 100 μm for b-d and f-h.

Figure 2. Association of myeloperoxidase (MPO) with sIA wall degeneration and inflammatory cell infiltration. **a**, The extent of MPO (MPO score) in the saccular intracranial aneurysm (sIA) walls associated with wall type, classified according to previously published criteria (7), where type A represents a wall with intact endothelium and linearly organized smooth muscle cells (SMCs); type B represents a thickened wall with disorganized SMCs; type C represents a hypocellular wall with organizing luminal thrombus; and type D represents a thin sIA wall with organized luminal thrombus. In this series, only two type-D sIAs were present, thus, these two sIAs were not included in the analysis due to their small number (13). Panel **b** shows a demonstrative MPO distribution (green) in the sIA wall and thrombus (pointing arrows). The negative control is the inset. Polymorphonuclear MPO^+ cells (arrowhead; the squared close-up image) were present in the sIA wall, representing the

presence of neutrophils. Mononuclear MPO^+ cells (star) were also present. MPO score correlated with the number of the following inflammatory cells: **c**, $CD163^+$ and **d**, $CD68^+$ macrophages, and **e**, $CD3^+$ T lymphocytes in the sIA wall. The number of inflammatory cells was counted in three standard-sized (each 0.613 mm^2) intensively stained areas (13).

Figure 3. High MPO score in the sIA wall correlated with the following markers of lipid accumulation: **a**, apolipoprotein B-100 (apoB-100, an integral protein in low-density lipoprotein, LDL) and **b**, oxidized lipid (ox-lipid, hydroxynonenal), but not with **c**, Oil Red O (ORO)-positive neutral lipid accumulation. **d**, Apolipoprotein A-I (apoA-I; the protein component of high-density lipoprotein, HDL) also correlated with MPO score. The lipid accumulation was measured as the percentage of positively stained area in the sIA wall (14).

Table 1. Association of myeloperoxidase (MPO) and matrix metalloproteinase-9 (MMP-9) scores with saccular intracranial aneurysm (sIA) wall type and rupture.

	Wall type				Bleeding status		
	A	B	C		Unruptured	Ruptured	
Variable	(n=9)	(n=12)	(n=11)	p value[†]	sIAs (n=16)	sIAs (n=20)	p value[†]
	Number of sIAs (%)				Number of sIAs (%)		
MPO				0.002[*]			0.003[*]
Score 1	2 (22)	3 (25)	0 (0)		5 (31)	0 (0)	
Score 2	5 (56)	4 (33)	0 (0)		7 (44)	4 (20)	
Score 3	2 (22)	3 (25)	3 (27)		1 (6.3)	7 (35)	
Score 4	0 (0)	2 (17)	8 (73)		3 (19)	9 (45)	
MMP-9				0.071[*]			<0.001[*]
Score 0	2 (22)	3 (25)	0 (0)		4 (25)	1 (5)	
Score 1	6 (67)	3 (25)	2 (18)		10 (63)	2 (10)	
Score 2	1 (11)	2 (17)	3 (27)		1 (6.3)	6 (30)	
Score 3	0 (0)	4 (33)	5 (46)		1 (6.3)	9 (45)	
Score 4	0 (0)	0 (0)	1 (9)		0 (0)	1 (5)	

[*]$p \leq 0.05$ considered significant.

[†]Fisher's exact test used, proportions are given.

Table 2. Correlation of myeloperoxidase (MPO) expression to clinical risk factors of saccular intracranial aneurysm rupture, and plasma lipid levels.

	MPO Score				
Variable	1 (n=5)	2 (n=11)	3 (n=8)	4 (n=12)	p value*
Population	Finnish	Finnish	Finnish	Finnish	
Hypertension† n (%)	4 (80)	2 (18)	2 (25)	4 (33)	0.141
Age‡	55 (49-66)	54 (29-67)	63 (41-87)	47 (24-74)	0.239
Size (mm)‡					
Base	3.6 (3.0-5.0)	4.0 (2.5-8.0)	4.5 (3.0-11.0)	3.0 (1.6-5.5)	0.288
Fundus lenght	5.5 (4.7-12.5)	6.5 (5.0-18.6)	9.5 (4.0-12.5)	6.0 (2.2-10.0)	0.317
Fundus width	5.3 (3.0-8.5)	5.0 (3.0-13.0)	7.3 (3.5-12.5)	4.8 (3.5-7.0)	0.372
Earlier subarachnoid hemorrhage†	1	0	1	0	0.124
Site†§					0.618
MCA (n=21)	3	6	5	7	
AcomA (n=7)	2	3	1	1	
PcomA (n=3)	0	2	1	0	
ICA (n=3)	0	0	1	2	
Other (n=2)	0	0	0	2	
PHASES Score‡‖	13 (8-14)	10 (7-13)	13 (8-14)	7 (5-15)	0.019*
Current smoking† (%)	4 (80)	7 (64)	4 (50)	3 (25)	0.161
Serum cholesterol value (mmol/L)‡					
Total cholesterol	5.4 (4.2-7.1)	5.6 (4.3-5.9)	5.7 (4.8-6.6)	5.4 (3.8-6.2)	0.626
LDL-cholesterol	2.8 (2.2-4.8)	2.9 (2.1-3.9)	2.7 (2.2-4.3)	2.6 (1.7-3.9)	0.707
Triglyserides	1.5 (1.1-2.7)	1.2 (0.6-2.2)	0.9 (0.6-5.4)	2.0 (1.0-3.5)	0.563
HDL-cholesterol	1.5 (1.1-1.7)	1.8 (1.5-2.0)	2.1 (0.9-3.0)	1.5 (1.3-1.7)	0.237

*$p \leq 0.05$ considered significant.

†Fisher's exact test used, proportions are given.

‡Kruskall Wallis test used, median and range are given for continuous variables.

[§]MCA, middle cerebral artery; AcomA, anterior communicans artery; PcomA, posterior communicans artery; ICA, internal carotid artery; Other, one posterior inferior cerebellar artery and one ophtalmic artery sIA.

[‖]Population, Hypertension, Age, Size, Earlier SAH, and Site (27).